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In vivo selection of lethal mutations reveals two functional domains in arginyl–tRNA synthetase

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ABSTRACT

Using random mutagenesis and a genetic screening in yeast, we isolated 26 mutations that inactivate *Saccharomyces cerevisiae* arginyl–tRNA synthetase (ArgRS). The mutations were identified and the kinetic parameters of the corresponding proteins were tested after purification of the expression products in *Escherichia coli*. The effects were interpreted in the light of the crystal structure of ArgRS. Eighteen functional residues were found around the arginine-binding pocket and eight others in the carboxy-terminal domain of the enzyme. Mutations of these residues all act by strongly impairing the rates of tRNA charging and arginine activation. Thus, ArgRS and $tRNA^{Arg}$ can be considered as a kind of ribonucleoprotein, where the tRNA, before being charged, is acting as a cofactor that activates the enzyme. Furthermore, by using different $tRNA^{Arg}$ isoacceptors and heterologous $tRNA^{Asp}$, we highlighted the crucial role of several residues of the carboxy-terminal domain in tRNA recognition and discrimination.

Keywords: aminoacyl–tRNA synthetase; genetic screen; kinetic analysis; mutants; substrate recognition

INTRODUCTION

Aminoacyl–tRNA synthetases (ARS) represent an outstanding example of a family of enzymes catalyzing the same reactions yet with slight differences of specificity, with respect to both the amino acid and tRNA. These enzymes catalyze two reactions, the first being the activation of the carboxyl group of the amino acid, resulting in formation of an enzyme-bound aminoacyl adenylate. In a second step, the activated amino acid is transferred to a specific tRNA with the formation of an energy-rich ester linkage at the 3′ terminal hydroxyl position. These enzymes exhibit high specificity during both reactions, as even in case of error during the enzymatic process, they can proofread the incorrect products or intermediates at the pretransfer or posttransfer level (Fersht, 1977).

Arginyl–tRNA synthetase (ArgRS) has a peculiar position among ARS. A striking property it shares with GluRS and GlnRS (all three being monomers) is the requirement of its cognate tRNA for catalysis of the ATP/PPi exchange occurring in the first reaction step. This has been an argument in favor of a mechanism without the formation of aminoacyl adenylate as a necessary intermediate (Loftfield & Eigner, 1969). Other authors came to the conclusion that despite this tRNA requirement, ArgRS may form arginyl-adenylate as an intermediate (Fersht et al., 1978). But until now, the true mechanism has not been clearly elucidated, maybe simply because the intermediate has not been found by usual methods in the absence of tRNA.

In the present study we have used a genetic approach to identify the important functional elements of yeast ArgRS. Random mutagenesis was used to screen the whole molecule. This results in the identification of crucial positions in the enzyme structure involved in catalysis, substrate binding, and discrimination. This approach appears to be an efficient way to identify amino acid side chains that are important for protein function, folding, and stability.

RESULTS

Gene cloning and characterization

The yeast genome contains two gene products presenting important homologies with *Escherichia coli* ArgRS (Mewes et al., 1997). It was reported that ORF

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Abbreviations: 5-FOA: 5-fluoroorotic acid; ArgRS: arginyl–tRNA synthetase; ARS: aminoacyl–tRNA synthetase; *RRS1*: ArgRS gene.

YHR091c encodes mitochondrial ArgRS (*MSR1*) (Tzagoloff & Shtanko, 1995) whereas ORF YDR341c encodes cytoplasmic ArgRS (*RRS1*) as shown by peptidic sequencing of purified enzyme (Gangloff et al., 1976; Sissler et al., 1997). We have further characterized the YDR341c product, located on chromosome IV, by disrupting the *RRS1* gene. The DNA fragment encoding *RRS1* was PCR amplified from genomic DNA, then the *HIS3* gene was cloned into *RRS1* in replacement of a DNA fragment encoding 383 internal amino acids, and the resulting linear fragment (Δ*rrs1::HIS3*) was introduced into the yeast strain YAMB4. The tetrads resulting from the meiosis and sporulation were dissected: two spores were unable to grow whereas the two growing spores were His^-. This was the first evidence showing that *RRS1* is an essential gene. If transformed with a rescuing plasmid encoding *RRS1* (pAL4: Ura^+, $Ade3^+$, $RRS1^+$), the disrupted diploid gave, after segregation, four viable spores. Two of them were His^- and were able to grow on 5-fluoroorotic acid (5-FOA) medium, whereas the two others were His^+ and 5-FOA sensitive (5-FOA^s), demonstrating that, when disrupted for *RRS1*, the yeast cells cannot lose the rescuing plasmid encoding *RRS1*. We concluded that *RRS1* is an essential gene.

This result also shows that the mitochondrial ArgRS encoded by *MSR1* cannot substitute for the absence of the cytoplasmic enzyme. This can be attributed to differences in the mode of tRNA recognition or to the fact that the active form of the mitochondrial isozyme is perfectly targeted to the mitochondrial compartment. In the cytoplasm, mitochondrial proteins are usually translated as inactive precursors presenting N-terminal mitochondrial localizing signals. The precursors are only activated after import and maturation in the organelle (Gasser & Hay, 1983; Hay et al., 1984). Most of the aminoacyl–tRNA synthetases exists as two isotypes encoded by nuclear genes. One gene codes for the cytoplasmic enzyme whereas the other one codes for the mitochondrial precursor. However, exceptions are found with the histidine and valine systems. The cytoplasmic and mitochondrial isozymes are synthesized from two sets of transcripts differing only by the 5′-end length. The initiation of the translation starts on two in-frame translation start sites located several dozen base pairs apart. Initiation from the longer transcript generates the precursor of the mitochondrial form, whereas translation from the shorter transcript results in the synthesis of the cytoplasmic enzyme (Natsoulis et al., 1986; Chatton et al., 1988).

Selection of lethal mutants of ArgRS

RRS1 is an essential gene, as shown by the inability of spores disrupted for *RRS1* to grow. By introducing a copy of *RRS1* (on pAL4: Ura^+, $Ade3^+$, $RRS1^+$) before meiosis, we can rescue the disrupted spores and thus build haploid strains disrupted for *RRS1* and maintained by a plasmidic copy of *RRS1*. We used this property to design a genetic approach for selection of ArgRS mutants affected in their enzyme function (Fig. 1). For the recipient yeast cell, a genetic background ($Ade2^-$, $Ade3^-$) was chosen to give a red pigmentation when rescued by the $Ade3^+$ vector (pAL4) and grown on medium containing limiting amounts of adenine (see Materials and Methods). Moreover, the presence of *URA3* in this vector confers a sensitivity to 5-FOA to the transformed cells. The screening procedure is based on the exchange of *RRS1* carried by pAL4 for another *RRS1* carried by pRS314 vector (Trp^+, $RRS1^+$). This exchange can occur when the second *RRS1* encodes a native enzyme and when the cells are spread on adequate growth medium. The exchange of pAL4 by pRS314-*RRS1* is followed by a loss of red pigmentation (colonies appear white/red sectored: $Sect^+$), due to *ADE3* loss, and by a 5-FOA resistance, due to *URA3* loss. In this way, it becomes possible to select, in a library of mutated *RRS1* genes carried by pRS314, the mutated genes that encode inactive ArgRS. These clones will appear red and nonsectored (red, $Sect^-$, and $5FOA^s$), as the rescuing pAL4 vector cannot leave the transformed cell. Thus, the selection of the lethal *RRS1* consists of a visual examination of isolated colonies. In this way, we screened 20,000 colonies cotransformed by pAL4 and by the mutated library pRS314-*RRS1*. We found 80 red, $sect^-$ colonies, unable to lose the pAL4 rescuing vector. From these clones we extracted 74 Trp^+ plasmids, and confirmed the red $Sect^-$ phenotype by reintroducing them into the YAL4 strain. Then, mutated vectors pRS314-*RRS1* were purified and the entire *RRS1* alleles and flanking regions were sequenced by seven primers.

Identification of the lethal mutations

We sequenced 74 *RRS1* alleles encoding lethal ArgRS. As expected, more than half of the mutated ArgRS genes were interrupted in their ORF before the normal TAA stop codon. We found 32 genes containing missense mutations, three of them were found in duplicate (SL409, AV450, and HY559). Some mutations were found as single mutants or associated with a second mutation (VI88+GD161; GD202+GE246; EK294+GN413; DN351+AV571; AV321+AV372; GD403+RT477; HY493+SL498). These double mutants required separation of the mutations to test the effects of the individual mutations. We used gene fragment replacements and site-directed mutagenesis to build the single mutants. In all cases, the lethal effects were found to be the consequence of a single mutation in *RRS1*. Thus, 26 different single mutations were identified (Table 1).

Taken together, most of the mutations identified in the lethal genes were base transitions (65 occurrences) consistent with the mutagenic properties of hydroxyl-

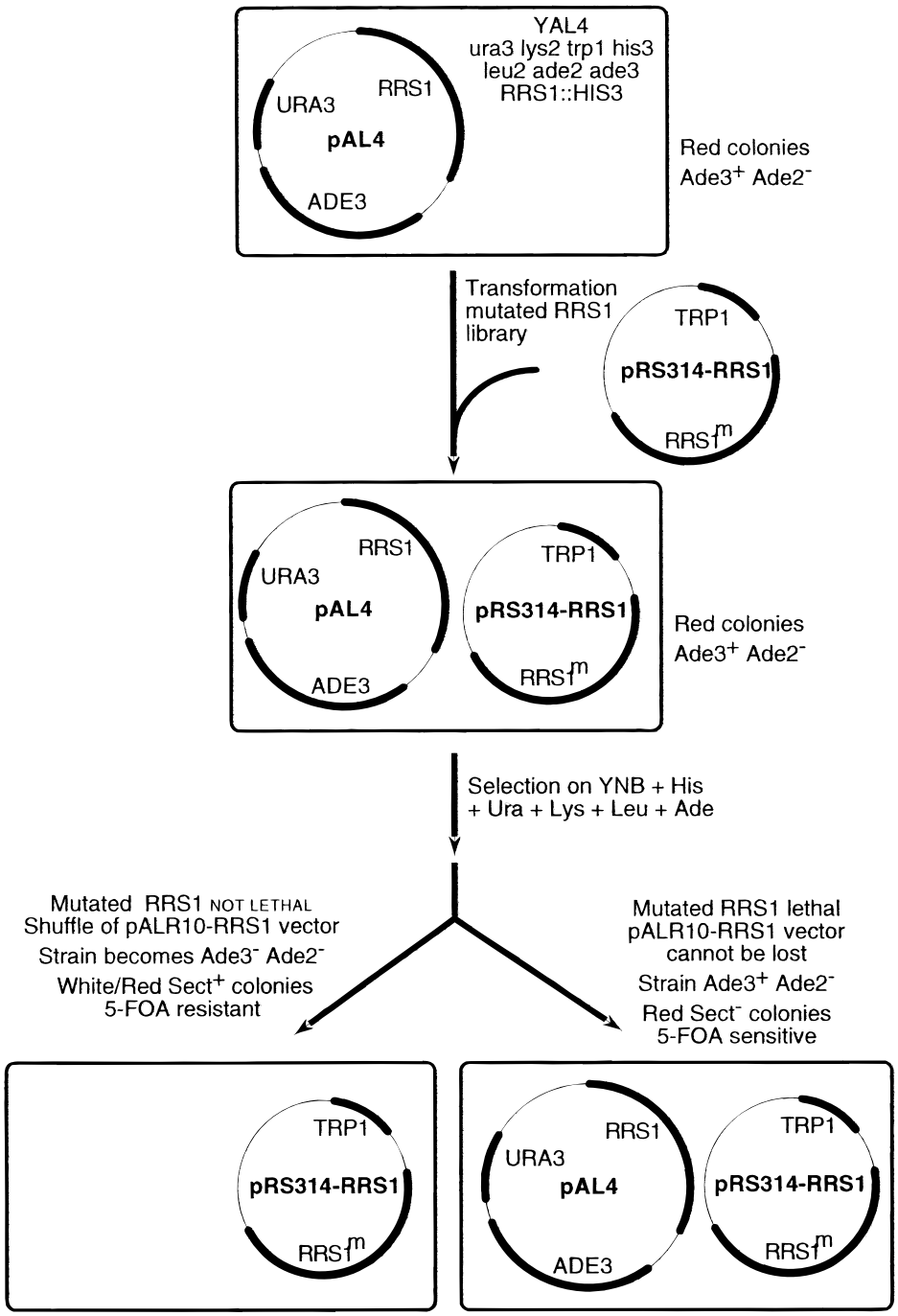

FIGURE 1. Schematic representation of the genetic screening procedure used for the selection of the lethal ArgRS genes. The *RRS1*-disrupted strain YAL4 (rrs1::*HIS3*), rescued by pAL4 (*RRS1*, *ADE3*, *URA3*), was transformed by the mutated *RRS1* library (pRS314-*RRS1*^{m}). The resulting yeast strain is red according to the Ade3^{+} Ade2^{-} phenotype. In the presence of an active ArgRS encoded by pRS314-*RRS1*^{m}, pAL4 can be lost and the resulting strain becomes Ade3^{-} Ade2^{-} (white/red sectoring colonies). In the presence of an inactive *RRS1*^{m} (lethal *RRS1*), pAL4 cannot be lost, and the colonies remain red nonsectored (red Sect^{-} colonies).

amine. We also found 11 transversion events, 6 deletions, and 1 insertion. Chemical mutagenesis tends to limit the selection of identical clones, a significant advantage when compared to PCR methods or to mutator strains that often lead to amplification of the same mutations.

Lethal mutations are clustered in two regions of the protein

The crystallographic structure of class I yeast ArgRS has recently been determined at 2.75Å resolution in the presence of arginine (Cavarelli et al., 1998). The

TABLE 1. Kinetic parameters of mutated ArgRS.

Enzyme	Aminoacylation tRNA tot (%)	Aminoacylation tRNA Arg2 (%)	K_m tRNA Arg2 (μM)	K_m ATP (mM)	Exchange (%)	Arginine (μM)
Native	100	100	0.34	1.33	100	5
Catalytic site						
AG160	3	0.4	0.588	100	0.06	4.5
GD161	<0.02	<0.02	nm[a]	nm	nm	nm
SF165			not overproduced			
GD190	<0.02	0.02	0.769	nm	nm	nm
GD197			not overproduced			
GD202			not overproduced			
EK294	<0.02	<0.02	nm	nm	nm	nm
TI349	20	99	0.217	0.425	10	2.4
RK350	11.5	2.2	0.364	4.6	0.7	10
DN351	0.7	0.05	0.323	10	0.06	28.5
DH351	0.5	0.4	0.7	45	0.35	13.3
AT372	0.09	0.2	0.222	100	0.04	1.8
AV372	1.5	0.55	0.167	170	0.1	1.3
GN403			not overproduced			
GC403	1.8	1.4	0.37	20	0.2	2.1
GD403			not overproduced			
SL409	6.5	7	0.424	4	1.4	3.3
RT411	0.8	0.04	0.2	42	nm	nm
Carboxy-terminal domain						
PL446	3	6.7	0.541	2.3	0.85	5.5
AV450	3.5	28	0.254	2.5	1.5	3.4
GS483	8.7	34	0.339	12.5	3.6	6.7
YH491	3.7	18.5	0.385	nm	2.5	6.25
SL494	0.3	23	0.556	0.5	3	4.25
RM495	0.8	1.3	0.403	7.5	2.3	4.3
SL498			not overproduced			
HY559	3.5	6.8	0.481	12.5	6	20

The activities are expressed as percentages relative to the rates of the wild-type enzyme, which are the following: aminoacylation of $tRNA^{total}$: 1.87 s^{-1}, aminoacylation of $tRNA^{Arg2}$: 3.96 s^{-1}, aminoacylation of $tRNA^{Arg3}$: 2.49 s^{-1}, ATP-PPi exchange reaction: 20.26 s^{-1}.Parameters strongly affected are shaded in dark grey, parameters moderately changed are shaded in light grey.

[a]nm: non measurable.

protein is essentially composed of five domains. The active site of the molecule is built as a Rossmann fold, on which are attached two additional domains (Add-1 and Add-2) at the N- and C-terminus respectively. Two domains (Ins-1 and Ins-2) are inserted in the catalytic core. We found 18 lethal mutations spread along the catalytic core and centered around the arginine binding site. A second cluster of mutations contains eight mutations located in the Add-2 domain, that is, the C-terminal helical domain (Fig. 2). This domain is structurally very similar to the C-terminal domain of the tryptsic fragment of *E. coli* MetRS, which has been shown to be involved in the binding and recognition of $tRNA^{Met}$ anticodon loop (Brunie et al., 1990; Ghosh et al., 1990, 1991; Meinnel et al., 1991; Kim et al., 1993b; Schmitt et al., 1993). The center of this second cluster is ~30 Å from the arginine molecule present in the active site.

Some of the selected mutations affect the class I signature sequences. In the HIGH-like sequence ($HA_{160}G_{161}H$), the following two mutations were found: AG160 and GD161. Natural variations are observed in ArgRS sequences at position 160 (Ala, Val, Leu, Ile, and Met, but no Gly) whereas residue Gly161 is strictly conserved in all sequenced ArgRSs. In the KMSKS-like sequence ($GMS_{409}TR_{411}$ sequence in the yeast enzyme) we isolated the following two mutations: SL409 and RT411. Residue 411 is a strictly conserved Arg, whereas at position 409, half of the ArgRS sequences contain a Lys residue (an Arg residue is also observed in *Arabidopsis thaliana* ArgRS). Other lethal mutations affecting invariant residues are EK294, DH351, DN351, YH491, and RM495. Seven lethal mutations (SF165, GD190, GD197, TI349, GC403, GD403, GN403, and GS483) concern residues conserved in a subclass of ArgRS containing the eukaryotic enzymes as well as some bacterial enzymes (*E. coli, Yersinia pestis, Enterobacter faecalis, Neisseria meningococus, Neisseria gonorrhoeae, Haemophilus influenzae, Pseudomonas aeruginosa*). Eight mutations affect residues presenting important natural variations (GD202, RK350, AT372,

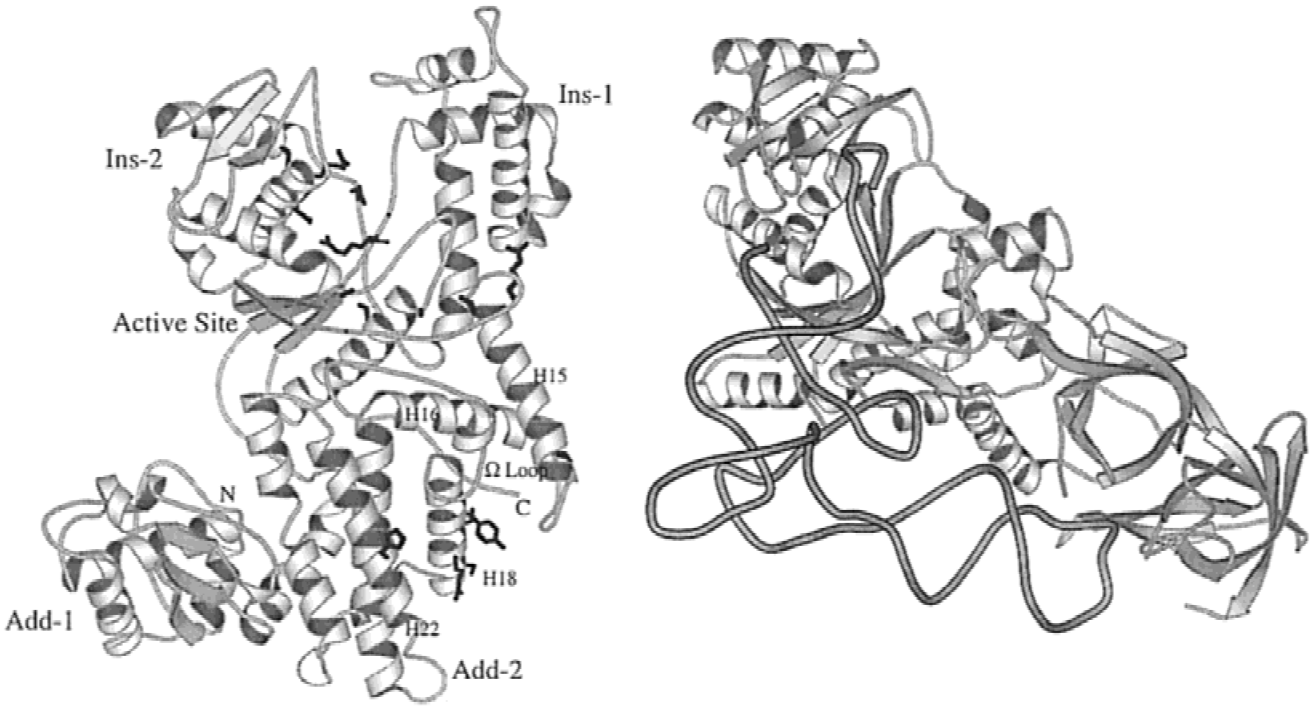

FIGURE 2. MOLSCRIPT representation (Kraulis, 1991) of two class I tRNA synthetases. ArgRS is shown on the left with the lethal mutations selected in the active site (top) and carboxy-terminal domain (bottom). GlnRS in complex with $tRNA^{Gln}$ is shown on the right (Rould et al., 1989). ArgRS and the GlnRS-$tRNA^{Gln}$ complex are oriented with respect to their Rossmann folds; thus, if a similar mode of tRNA binding were to occur in the ArgRS-$tRNA^{Arg}$ complex, one would predict interactions with the N-terminal domain (Add-1) and C-terminal domain (Add-2) (with the D-loop and anticodon loop of $tRNA^{Arg}$, respectively).

AV372, PL446, AV450, SL494, and HY559). Here we want to mention that a Lys350 and a Leu446 are naturally found in some species.

Heterologous expression of the mutated proteins

To isolate enough purified protein of ArgRS mutants for kinetic investigations, we cloned the mutated *RRS1* into pTrc99B. We replaced its 1908-bp *Eco*RI-*Xho*I fragment encoding 460 residues of the native enzyme with the corresponding fragment of the mutated proteins. Then we grew and induced the cells in the presence of the helper $tRNA^{ArgU}$ as described previously (Cavarelli et al., 1998). The main advantage of this heterologous expression in *E. coli* is that the mutants are free of native yeast ArgRS contamination. Moreover, the mutants are easily separated from the *E. coli* ArgRS on the hydroxyapatite column (the *E. coli* enzyme is eluted below 50 mM of potassium phosphate buffer, the yeast enzyme above 150 mM of the same buffer). Some mutated ArgRSs were isolated from this column despite the fact they were completely inactive; this was possible because no other bacterial proteins are eluted at the same phosphate molarity, and because of the high reproducibility of the HPLC fractionation. In the case of six clones, no expression products could be detected. Protein instability, important degradation, or low overexpression could explain this absence of protein in the crude extracts.

Kinetic analysis

Aminoacylation and pyrophosphate exchange reactions were measured at the steady-state level. Dissociation constants (K_m) for ATP and $tRNA^{Arg3}$ were measured in the aminoacylation reaction, and K_m for arginine was measured in the pyrophosphate exchange reaction. Results are shown in Table 1. Only two of the purified proteins present nonmeasurable aminoacylation. They correspond to the strictly invariant residues Gly161 and Glu294. The other mutated proteins display reduced aminoacylation rates when tested with total tRNA preparations (mimicking the in vivo conditions of tRNA selection and aminoacylation). Some of these values are increased up to the wild-type enzyme values when tested with pure $tRNA^{Arg2}$, thus arguing for competition events by noncognate tRNAs for the mutated tRNA-binding site.

Important decreases of the ATP-binding strength were observed for several mutants: AG160, in the HIGH-like sequence, RT411, in the KMSKS-like sequence, DH351,

a key residue for arginine binding, AT372, and AV372, in close proximity to the place where the adenine ring of the ATP molecule should be present (position deduced by superimposing the ArgRS and GlnRS Rossmann folds, the latter residue being present in the ternary GlnRS-tRNA^{Gln}-ATP complex). Several mutations located in the carboxy-terminal domain of the enzyme also induce significant effects on ATP binding (GS483 and HY559).

Only moderate effects on the K_m for arginine were observed. The highest decrease of affinity occurs with residue Asp351, which interacts with the guanidinium moiety of the arginine molecule. Another mutant presenting a moderate effect on the arginine is found at position 559 (HY559).

We could not detect any mutant having a modified affinity for tRNA^{Arg}. The mutations suspected to be involved in tRNA binding affect both the aminoacylation and the activation rates (Table 1). This is consistent with the absolute prerequisite of tRNA^{Arg} for the first step of activation of arginine. We also tested the aminoacylation properties of ArgRS mutants for two tRNA^{Arg} isoacceptors (tRNA^{Arg2} and tRNA^{Arg3}) and the heterologous tRNA^{Asp}. Thus, we observed different effects on the tRNA^{Arg} recognition properties of mutants PL446 and SL494 (Fig. 5), whereas another mutant (YH491) exhibits a significant increase of the mischarging rate of tRNA^{Asp} (Fig. 6).

DISCUSSION

We have isolated a set of lethal mutations in the gene encoding cytoplasmic yeast ArgRS. The effects of the mutations on the catalytic properties of the enzyme were determined and could be interpreted on the basis of the crystallographic data available on ArgRS and some other class I synthetases (MetRS, IleRS, GlnRS-tRNA^{Gln} complex).

A first remark concerns the location of the mutations. Eighteen mutations were found in the catalytic Rossmann fold domain, eight in the carboxy-terminal helical domain, and no mutation was found in the amino-terminal domain (Fig. 2). This domain is composed of 142 residues and is idiosyncratic to the ArgRS enzymes. It may be involved in the binding of the dihydro U loop of the tRNA (Cavarelli et al., 1998) where is found the major identity element in the *E. coli* system (residue A20) (McClain & Foss, 1988; McClain et al., 1990). In the yeast system, we do not know if the absence of selection of lethal mutations in this domain is correlated to the absence of conservation of residue A20 in the four different yeast tRNAs^{Arg}. Structural studies on the yeast ArgRS: tRNA^{Arg} complex, which are currently in progress, will give some insights on this point (B. Delagoutte & J. Cavarelli, to be published).

Eighteen essential positions were identified in the catalytic domain

The yeast ArgRS has been crystallized in the presence of arginine (Cavarelli et al., 1998). The binding of this substrate has been described as involving at least seven different side chains located in a crevice formed between the two halves of the Rossmann fold. The cocrystal with ATP has not yet been obtained, but we know from extensive studies realized on tyrosyl- and methionyl-tRNA synthetases that the HIGH and KMSKS class I consensus sequences are involved in ATP binding and stabilization of the transition state (Carter et al., 1984; Leatherbarrow et al., 1985; Brick et al., 1989; Mechulam et al., 1991; Schmitt et al., 1994, 1995). Structural information on these enzymes was used to interpret the deleterious effects of mutations on the ArgRS properties (Fig. 3, Table 1).

Among the 18 lethal mutations selected in the catalytic site, five could not be overexpressed in *E. coli*. They consist essentially of Gly and Ser residues that were mutated in larger and sometimes polar residues (GD, GN, SF). These changes may result in loss of flexibility and steric hindrance that could generate structural effects, instability, and, hence, degradation in *E. coli*. We do not know if, in the yeast cell, the same phenomenon may be responsible for the lethal phenotype, or if these mutant proteins are correctly folded but catalytically deficient.

Four lethal mutations were found in the consensus-like HIGH and KMSKS sequences and two affected residue 351, identified as one of the main partners in the recognition of the arginine substrate. These mutations induced important decreases in ATP affinity and in catalytic activities, but only moderately affected the arginine affinity. This absence of drastic variation of the K_m for arginine may be correlated to the high density of interaction observed in the ArgRS-arginine binary complex (Cavarelli et al., 1998). Eight residues are involved in recognition of the amino acid through H-bonds and salt bridge interactions. This accounts for the high affinity for arginine ($K_m = 5\ \mu$M) and its moderate change upon mutation of one of these residues: DH351 or DN351. On the other hand, one may not exclude that these mutations may rearrange the network of interaction with arginine, but in a way leaving unchanged the global interaction strength with this substrate. Moreover, the important k_{cat} decrease reveals a role of Asp351 in stabilization of the transition state of the reaction. Thus, residue 351 appears to be involved in initial binding of the arginine molecule and in stabilization of the transition state of the activation reaction.

Concerning the high affinity measured for arginine, one may see a relationship with arginine metabolism. Arginine presents important structural similarities with several metabolic intermediates that may represent potential competitors for the arginine-binding site of

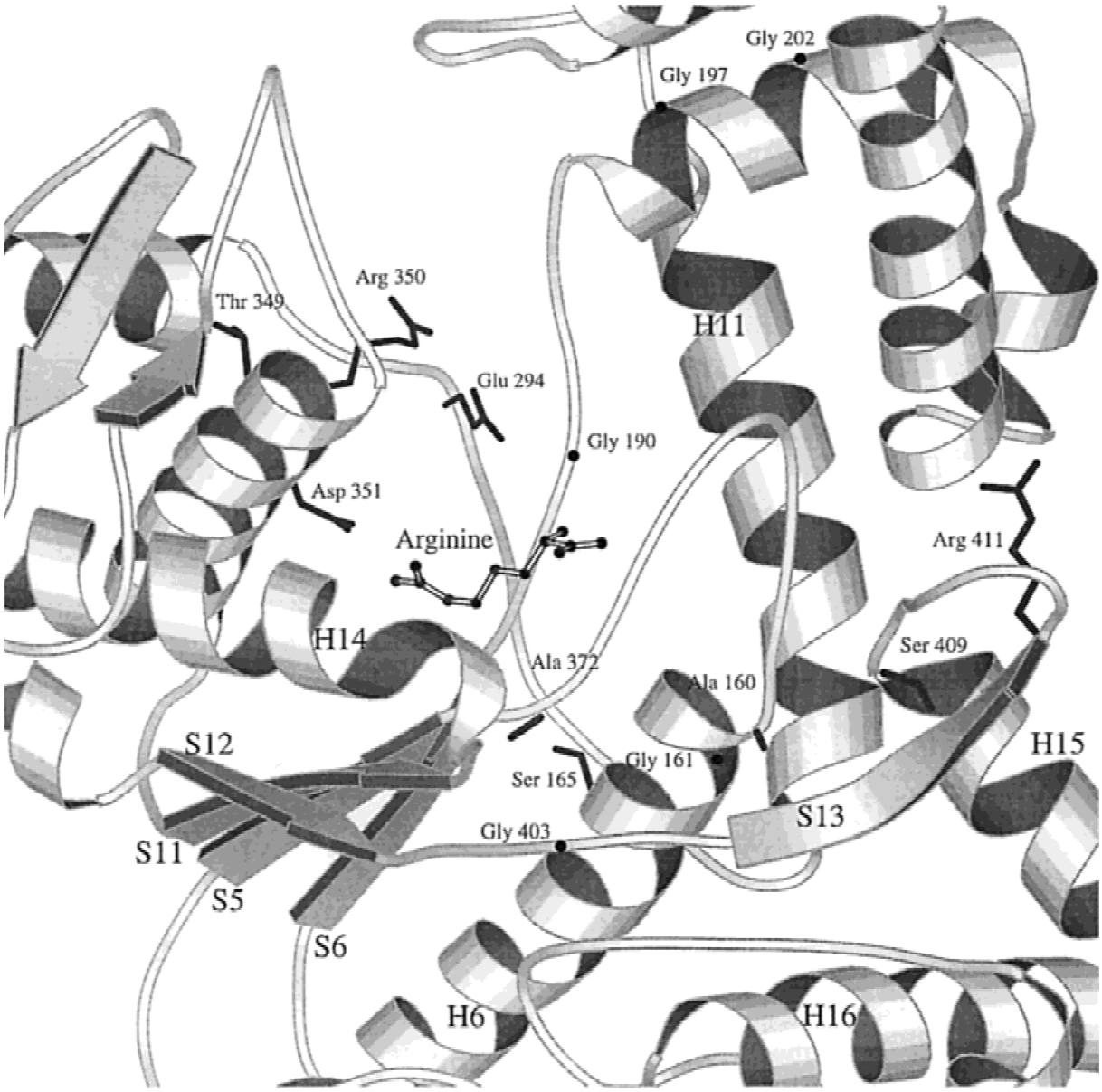

FIGURE 3. General view of the active site of ArgRS showing the mutated positions and the arginine molecule. Numbering of strands and helices is according to Cavarelli et al. (1998).

ArgRS. To avoid such phenomena, affinity of ArgRS for arginine may have increased to create very high specificity. It results in a K_m value for arginine 200 times lower than the arginine concentration within the cell (about 1 mM; Messenguy et al., 1980). Metabolic intermediates and analogs have been tested by several authors: L-isomers of ornithine, citrulline, homocitruline, nitroarginine, arginosuccinate, sulfaguanidine lysine, and α-amino-γ-guanidinobutyric acid have little or no effect on the formation of arginyl–tRNA (Boman et al., 1961; Allende & Allende, 1964; Charlier & Gerlo, 1976). An inhibiting competition with homoarginine without esterification of tRNA has been observed (Mitra & Mehler, 1967). Among the analogs tested by Allende & Allende (1964) only canavanine competitively inhibited arginyl–tRNA formation ($K_i = 4.5 \times 10^{-5}$ M) and is itself attached to tRNA in place of arginine. The inhibitory effect of canavanine on cell growth can therefore be partly explained by its involvement in protein synthesis and protein structure.

In close proximity to residue 351, and on the same helix, mutations TI349 and RK350 induce lethality. Whereas residue 349 is oriented towards insertion domain 2 (Ins-2) on the opposite side of the active site, residue 350 is oriented towards the active site cavity at bonding distance to residue 294 (see below; Fig. 3). Mutation TI349 could modify the conformation of Ins-2 by changing the interactions with residues 338, 339,

and 345. It has previously been proposed that Ins-2, together with Ins-1, may clamp the $tRNA^{Arg}$ acceptor arm (Cavarelli et al., 1998); we show here with the TI349 mutant that the Ins-2 domain may also be involved in $tRNA^{Arg}$ recognition. Indeed, when assayed with crude tRNA, the mutant catalyzes the aminoacylation with a reduced rate (20% of the native rate), in contrast to the full activity measured with pure $tRNA^{Arg2}$ (Table 1). This kind of behavior was already observed in synthetases mutated in their tRNA-binding sites and was interpreted as competition by heterologous tRNAs resulting from a relaxation of tRNA-specific recognition (Ador et al., 1999; Eriani & Gangloff, 1999). Thus, the mutation TI349 probably changes the structure of the tRNA-binding site. It results in a competition of noncognate tRNAs for the altered binding site and a decrease in the aminoacylation rate. With pure $tRNA^{Arg}$, mutant TI349 also reveals an alteration of the activation efficiency, whereas the aminoacylation process looks intact, indicating that TI349 also affects the structure of the activation site itself. To summarize, TI349 may relax the recognition specificity of ArgRS for $tRNA^{Arg}$ and impair the amino acid activation site, an effect which may occur in two nonexclusive ways. First, the mutation TI349 could affect the structure of the activation site of the enzyme by an indirect effect. Second, the $tRNA^{Arg}$ incorrectly bound in the active site would be a less efficient cofactor and thus would promote the activation reaction less efficiently. Obviously, the $tRNA^{Arg}$ structural elements responsible for this activation should be located near the acceptor end. Several authors have shown that the acceptor ribose must be located in close proximity to the α-phosphate of ATP and the carboxylic group of arginine, and that periodate-oxidized $tRNA^{Arg}$ does not catalyze arginine-dependent ATP-PPi exchange (Godeau & Charlier, 1979). Thus, the ribose integrity has to be preserved for activation of arginine.

Mutants of the positions 372 and 403 were selected several times with the same or with different substitutions. This high frequency could reflect some hot spot of mutagenesis on the DNA or reflect the crucial importance of these residues. Residue Ala372 is located in a loop connecting strand S11 to helix H14. This loop can be well superimposed with the equivalent one found in the ternary complex GlnRS-$tRNA^{Gln}$-ATP. In GlnRS, the Ala372-equivalent residue (Thr230) is at bonding distance of the ATP molecule, between the 2′ OH of the ribose and the adenine ring of ATP. Thr230 is also closely connected with Phe233, which binds the terminal adenine ring of A76 (Rath et al., 1998). By analogy, the ArgRS loop where Ala372 is located could also play a crucial function, a hypothesis strongly supported by the important variations of ATP affinity observed for the two selected mutations. Interestingly, a Thr-mutation mimicking the Thr230 of GlnRS was selected, but the lethal effect observed suggests differences in the network of interaction with the substrates. The second residue mutated several times is Gly403. This residue is located between the two last strands, S12 and S13, of the Rossmann fold, a few residues before the KMSKS-like sequence (MSTR). In GlnRS, this domain is very near the ATP molecule, the residue equivalent to Gly403 being a Ser in that enzyme. In *Thermus thermophilus* MetRS (PDB #1A8H) and IleRS (Nureki et al., 1998) a Gly is found at that position. The fact that mutants GD403 and GN403 could not be overproduced strongly suggests that the mutations affect the structure of S12 and S13, thus resulting in unstable proteins, whereas the smallest substitution (GC403) is decreasing the catalytic activity of ArgRS and its affinity for ATP.

Mutations GD190 and EK294 are located at the bottom of the active site pocket, at the entrance of the Ins-1 and Ins-2 domains, respectively. The mutation of the Gly residue at position 190 could change the flexibility of the loop or create steric clashes. It could act directly on the adjacent invariant residue Asp191, whose structural role has been reported (Cavarelli et al., 1998). It stabilizes the interactions of strand S5 with strand S6 and the surrounding secondary structures. It is worth mentioning that the same peptide was identified as a potential proofreading site in MetRS (Kim et al., 1993a). Mutant EK294 affects one of the ArgRS invariant residues. Analysis of the structural superimposition ArgRS/GlnRS-$tRNA^{Gln}$-ATP suggests that Glu294 adopts a position very close to the ATP molecule. The activity of the enzyme was so low that we were unable to determine any catalytic parameter for this mutant, but a very strong dependence on high ATP and tRNA concentrations was observable.

Eight mutations are found in the carboxy-terminal domain

At its carboxy-terminal end, ArgRS exhibits an α-helical carboxy-terminal domain composed of about 200 residues folded in 10 helices and one strand (Fig. 4). A similar elongated domain is found in other class I synthetases like MetRS (Brunie et al., 1990; Konno et al., PDB #1A8H), IleRS (Nureki et al., 1998), and probably in other enzymes of the same subgroup of sequence homology (LeuRS and ValRS). With MetRS, superimposition of the corresponding Cα atoms of the two domains (291 residues) yields root mean square deviations of 2.48 Å (Konno et al., 1998, PDB #1A8H).

The recently solved IleRS-$tRNA^{Ile}$ complex reveals that this domain interacts with the anticodon loop of the tRNA molecule (Silvian et al., 1999). By analogy with these structural data, which agree with the functional data collected on MetRS, we would predict that the ArgRS's carboxy-terminal module may also be involved in tRNA binding. However, when compared with MetRS, some structural differences appear that may be explained by the fact that ArgRS has to accommodate

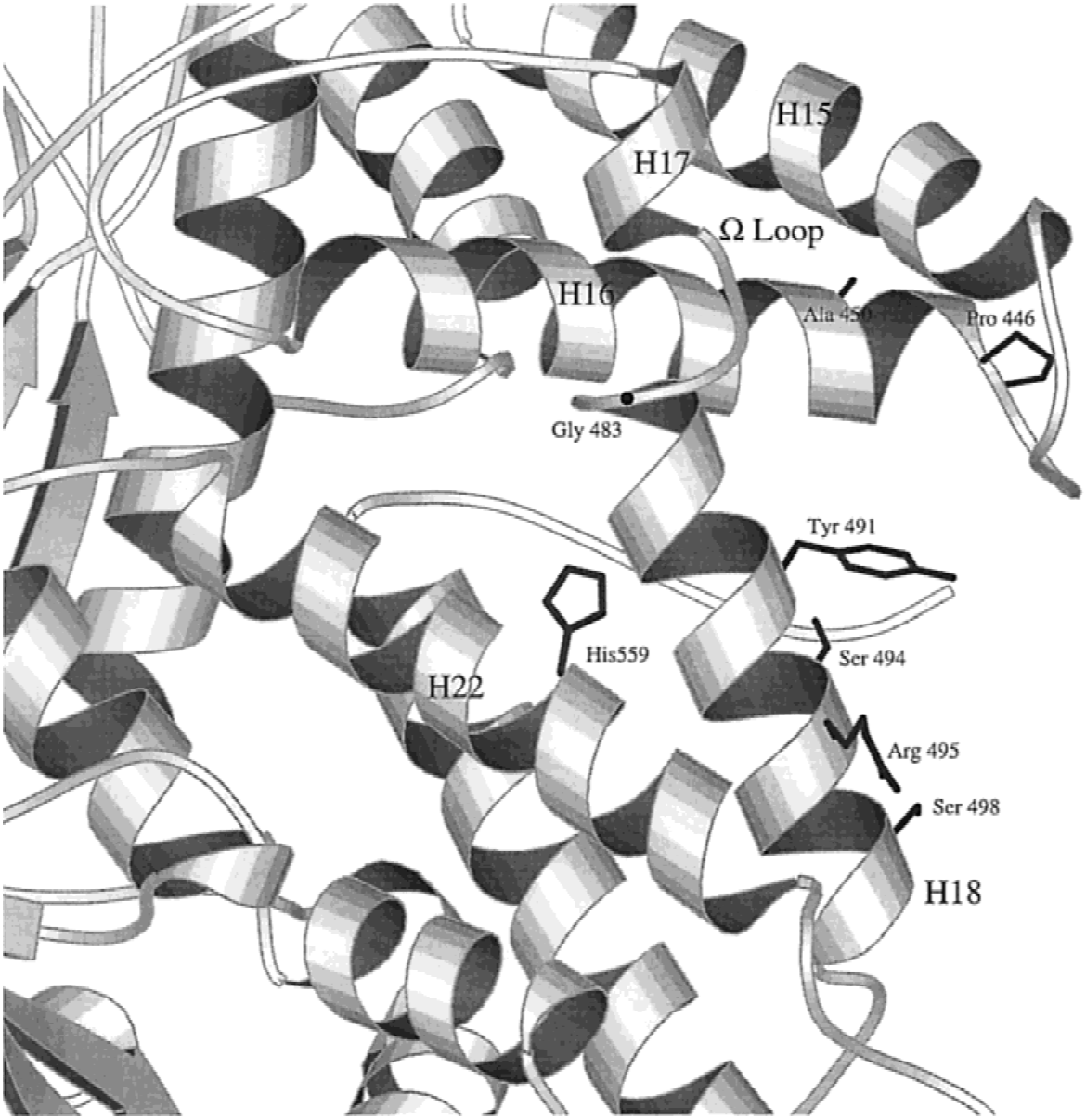

FIGURE 4. View of the carboxy-terminal module of ArgRS showing the mutated residues.

anticodon binding of different tRNA isoacceptors. MetRS recognizes elongator and initiator $tRNA^{Met}$ presenting the same anticodon (CAU), whereas yeast ArgRS recognizes four different $tRNA^{Arg}$ (anticodons: CCU, ICG, ^{mcm5}UCU, CCG), which would imply a more complex mechanism of tRNA discrimination. In yeast, ArgRS helices H15 and H16 (Figs. 1 and 4) following the catalytic domain are much longer than the equivalent ones in MetRS, thus extending their interaction capacity by more than 15 Å. Two lethal mutations were found in this extension (PL446 and AV450). It is remarkable that in MetRS, the end of the truncated protein folds back towards the catalytic module and seats in a spatial location equivalent to the ArgRS extension. Another difference between the two anticodon-binding domains concerns the H17 and H18 helices of ArgRS (residues 475–479 and 486–500), which present a striking structural difference when compared to MetRS. The two helices are almost continuous, as in MetRS, except that a small peptide (Ser480–Thr485) connects them by forming a protruding Ω loop at the surface of the protein (Cavarelli et al., 1998). This peculiar structure may be used by yeast ArgRS as a tRNA-recognition motif as suggested by the lethal mutation selected in this peptide (GS483). The equivalent unique MetRS helix (*H14*), together with the adjacent helix *H16*, forms the anticodon-binding site for $tRNA^{Met}$ (*H16* of MetRS = H22 of ArgRS; see Fig. 4). Several residues involved in $tRNA^{Met}$ binding or in suppression of amber $tRNA^{Met}$ have been characterized in these helices (Ghosh et al.,

1990, 1991; Meinnel et al., 1991; Kim et al., 1993b, 1993c), and residues that discriminate noncognate tRNAs have also been identified in helix *H16* (Schmitt et al., 1993). Our genetic approach allowed us to isolate four mutations in helix H18 (YH491, SL494, RM495, SL498) and one in H22 (HY559, the MetRS-*H16* equivalent). These data reinforce the idea that this module is also the anticodon-binding site for $tRNA^{Arg}$. Helices H15 and H16 are located 10–15 Å away, and therefore may also be suspected of participating in the binding of the anticodon loop.

Most of the mutant proteins could be overexpressed in *E. coli* and purified. Steady-state kinetics were performed to measure the exchange and tRNA charging rates. As expected with this enzyme that needs the homologous tRNA for the adenylation reaction, we did not obtain a mutant where the effects were only visible on the charging rate. The two reactions were simultaneously affected and in some cases the activation reaction was more severely impaired than the charging reaction. This addresses the problem of the $tRNA^{Arg}$ prerequisite for the arginine activation; our results clearly suggest that changing the conformation of the tRNA-binding site affects the activation of arginine. In the case of GlnRS, it has been proposed that the binding of the terminal adenosine would create the amino acid-binding pocket and thus make the activation reaction efficient. This was demonstrated by a mutational study showing covariation of the amino acid and $tRNA^{Gln}$-binding strengths (Ibba et al., 1996; Hong et al., 1996). Using glutaminyl-sulfamoyl-adenosine (QSI) as a stable analog of the glutaminyl adenylate, other authors proposed that the adenine ring and the ribose of A76 form van der Waals contacts with Phe233 and Tyr211, thereby positioning them and creating part of the glutamine-binding site (Rath et al., 1998). Together, Phe233 and Tyr211 form a hydrophobic lid that seals off the glutamine-binding site through van der Walls interactions with the aliphatic portion of glutamine. It has been mentioned by the same authors that residue Tyr211 is strongly conserved in all members of the class I synthetases, including ArgRS. In the case of ArgRS, no structural answer is yet available and it has not been established if the equivalent residue (Tyr347) plays the same function as in GlnRS. It binds the η-nitrogen atom of arginine cooperatively with Asp351 and Glu148 in the binary complex ArgRS-arginine, but unfortunately was not selected as a lethal mutant in our screen. However in the case of ArgRS, the mutations that inactivate the enzyme through the binding of the tRNA do not affect the arginine-binding strength. Of the seven lethal mutation analyzed in the anticodon-binding domain only one shows a moderate effect on the arginine affinity (HY559; Table 1). This corroborates results of Cavarelli et al. (1998) who observed, in obtaining the binary complex ArgRS-arginine, that tRNA binding is not a prerequisite for arginine binding. Comparatively, more mutants are affected in their ATP binding: GS483, RM495, and HY559 present moderate decreases in the ATP affinity. Thus, we could not observe amino acid affinity decreases as observed in the cases of GlnRS or TrpRS (Ibba et al., 1996); only important decreases in the catalytic rates were measured, probably resulting from an incorrect positioning of the tRNA in the active site. As ArgRS needs tRNA to activate the amino acid, this may explain why the amino acid activation step is always affected on mutation of the tRNA-binding site.

ArgRS mutants discriminate noncognate tRNAs by catalytic effects

Arginylation of $tRNA^{Arg}$ was tested in different tRNA contexts. When tested with total tRNA, a situation mimicking the in vivo cellular conditions, the highest measured charging activity only reaches 20% of the native activity (Table 1), a level which appears to be too weak to complement the disrupted *RRS1* strain. When tested with pure $tRNA^{Arg2}$, the catalytic rates are sometimes increased and reach the wild-type level for mutant TI349. Significant increases are also observed for mutants of the anticodon-binding domain: PL446, AV450, GS483, YH491, SL494, and HY559. This means that the mutated synthetases are still able to catalyze the arginylation reaction, but are unable to select and bind their homologous tRNA with high specificity in a crude tRNA preparation. Such competition phenomenon has already been observed for several synthetases. It is due to the fact that tRNA molecules are structurally very similar, thus rendering tRNA discrimination very subtle and sensitive to imbalances and competitions (Sherman & Söll, 1996; Ador et al., 1999; Eriani & Gangloff, 1999).

Among the mutants selected, mutant HY559 is of special interest because it occupies spatially the place of residue Asp449 in *E. coli* MetRS. This acidic residue, together with Asp456, maintains the correct balance between cognate and noncognate tRNA binding by rejecting noncognate tRNAs (Schmitt et al., 1993). However, in the case of MetRS, these residues do not affect the catalytic efficiency of aminoacylation of the authentic $tRNA^{Met}$, but they only act against noncognate tRNAs. ArgRS seems to play a more conventional role in tRNA binding, as judged by the effects on the catalytic rates when changed in Tyr. This example illustrates well how a similar structural framework can differentially be used by two different enzymes of a related subgroup.

Although the catalytic rates are strongly decreased, K_m measurements for $tRNA^{Arg2}$ revealed no significant variation among the 26 tested mutant proteins. Thus, our genetic selection demonstrates that the strongest effect of an inactivating mutation is on the stability of the transition state of the reaction, rather than on substrates' affinities in the ground state. Consequently, the

easier way to inactivate an enzyme is to decrease the stability of the transition state of the reaction, rather than to decrease the substrates' affinities. Moreover, it is important to note that cellular concentrations of substrates often can compensate for the K_m increases, and may negate the effect of the corresponding mutations, excluding them from the selection.

From our results it appears that the incorrect binding of the $tRNA^{Arg2}$ we have generated by mutation leads to a less-productive enzyme–substrate complex. Thus, the effect of the disrupted interaction is conveyed to the active site where the tRNA-dependent adenylation takes place, and induces a loss of stability of the transition state. That no significant variation of tRNA binding energy could be measured may be explained by the fact that the loss of one interaction may induce a repositioning of the tRNA with creation of new interactions compensating for the loss of binding energy. The final result is an incorrect positioning of the acceptor end of the tRNA in the transition state.

In the present study, about one third of the mutations we isolated were located in the anticodon-binding domain (8/26). In a previous study, we isolated 23 lethal mutations in the AspRS gene (Ador et al., 1999). Only one was located in the anticodon-binding site, where it induced a severe drop in $tRNA^{Asp}$ affinity and favored competition by the other noncognate tRNAs. But the activation rate of this mutant for aspartic acid was unchanged. This illustrates well the difference between the two categories of enzymes. ArgRS uses tRNA first as a cofactor, then as a substrate, its accurate binding being absolutely necessary for the activation of the amino acid. ArgRS and $tRNA^{Arg}$ can be considered as a kind of ribonucleoprotein, hence its high sensitivity to tRNA-binding changes, even when the latter are affecting distal regions of the molecule.

A functional connection between the anticodon-binding module and the active site

We know that conformational changes occur in the synthetases and/or tRNA upon complex formation. These should be particularly crucial in the arginine system where accurate tRNA binding is required for aminoacyl–adenylate formation. Functional binding of $tRNA^{Arg}$ and discrimination of noncognate tRNAs seems to depend only on identity element C35, located in the anticodon (Sissler et al., 1996) and 35 Å away from the active site. This raises the question of how the anticodon tRNA binding signal is transmitted to the active site. In the case of noncognate tRNA binding, some conformational changes and structural communication should prepare the active site for incorrect binding. It is reasonable to assume that the tRNA structure is the first affected by heterologous binding, and communication with the active site may occur via a tRNA molecular pathway. It would result in nonreactive binding. One may also speculate about internal enzyme communication when incorrect binding occurs. In this case, the conformational changes could be transmitted via the long helices of the anticodon binding domain (H21, H22, H23) to helix H6, located underneath the floor of the active site, which carries the "HIGH" signature motif (Cavarelli et al., 1998). Using these helices that are more than 30 Å long could be an easy way to transmit information to the active site located at a distance of 35 Å.

Some ArgRS mutants exhibit different tRNA recognition properties

To compare the tRNA recognition properties of the different ArgRS mutants we tested the aminoacylation properties of two $tRNA^{Arg}$ isoacceptors. We used the modified $tRNA^{Arg2}$ and $tRNA^{Arg3}$ purified from bulk tRNA (Kuntzel & Dirheimer, 1968; Weissenbach et al., 1972). These tRNAs correspond to the major $tRNAs^{Arg}$ in the yeast cell, whereas the two minor tRNAs, $tRNA^{Arg1}$ and $tRNA^{Arg4}$, are not present in high enough concentration to be purified. We used only modified tRNAs, because we know that native ArgRS exhibits a relaxed specificity for unmodified tRNAs like $tRNA^{Asp}$ (Perret et al., 1990). Figure 5 shows data measured for two lethal mutants. Whereas the native ArgRS shows comparable catalytic rates for the two isoacceptors, mutants PL446 and SL494 present different catalytic rates. PL446 is 4.3-fold more active with $tRNA^{Arg3}$ whereas SL494 charges $tRNA^{Arg2}$ 26-fold more efficiently. Obviously, these differences would imply that the tRNA-binding sites for the two tRNAs are at least partially distinct. In the ArgRS structure, the two mutations are located in the anticodon-binding domain in helix H16 (PL446) and helix H23 (SL494) at a 14-Å distance from each other. When examining the anticodon loop, the

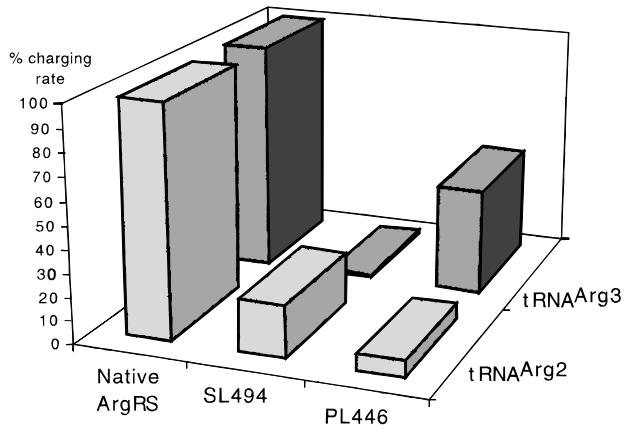

FIGURE 5. Histogram showing the changes in the tRNA recognition properties of two ArgRS mutants. The aminoacylation rates are expressed as percentages of native enzyme rate. K_m values for $tRNA^{Arg3}$ are the following: native, 0.645 μM; PL446, 0.24 μM; SL494, 0.29 μM.

main difference between the two tRNAs is at the anticodon level, where ICG and ^{mcm5}UCU are found for $tRNA^{Arg2}$ and $tRNA^{Arg3}$, respectively. If the central C35 is the major identity element of yeast $tRNA^{Arg}$, the recognition depends to a lesser extent on position 36 where U and G (found in the arginine isoacceptors) are more accepted than A and C residues (Sissler et al., 1996). It is also interesting to note that the native enzyme shows nearly the same affinity and catalytic efficiency for the two major isoacceptors, which would imply that a unique tRNA-binding site is used by the enzyme. To solve this apparent contradiction it may be hypothesized that the different tRNA-binding sites overlap to present the common recognition elements to the enzyme "determinants". Residue Pro446 would be part of the specific $tRNA^{Arg3}$ subsite whereas Ser494 would be more specific for $tRNA^{Arg2}$.

It has been known for a long time that yeast ArgRS mischarges $tRNA^{Asp}$ (Gangloff et al., 1973), but only recently has it been shown that this reaction can be amplified by two orders of magnitude with unmodified synthetic $tRNA^{Asp}$ (Perret et al., 1990). The molecular signal responsible for this specificity relaxation was later identified as the methyl group in position 1 of G37 (Pütz et al., 1994); this methyl group would act as an antideterminant in preventing efficient recognition of $tRNA^{Asp}$. We have used this ArgRS peculiarity as a test to explore the mischarging properties of pure $tRNA^{Asp}$ by the lethal mutants. Again we chose to work with fully modified $tRNA^{Asp}$ purified from bulk tRNA. Figure 6 summarizes the significant data we obtained. Mutant YH491 presents a tenfold increase in the $tRNA^{Asp}$ aminoacylation velocity. Again, a mutant of the anticodon-binding module is responsible for this change of tRNA recognition and with a catalytic increase in a heterologous tRNA. Mutation YH491 probably contributes by generating new contacts with aspartic tRNA that increase the stability of the transition state of the reaction. Tyr491 is located in helix H23 near Ser494, which shows an important imbalance in the ratio of $tRNA^{Arg2}$/$tRNA^{Arg3}$ aminoacylation (see above). Thus, the significant variations in the tRNA recognition properties induced by these four mutations (491, 494, 495, and 498) show that helix H23 contributes in a crucial way to specificity of the aminoacylation reaction.

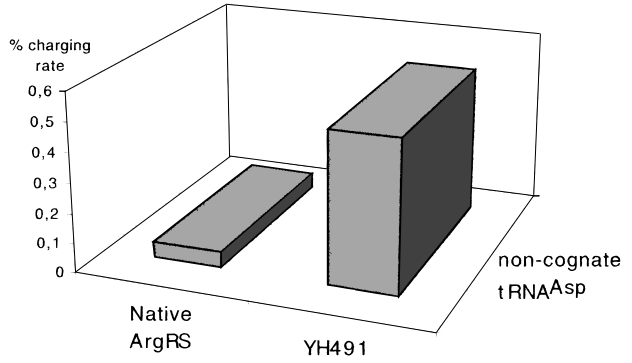

FIGURE 6. Histogram showing the increase of the noncognate $tRNA^{Asp}$ charging activity measured for mutant YH491, compared to the native enzyme activity. The aminoacylation rates are expressed as percentages of aminoacylation rate of $tRNA^{Arg2}$ by the native enzyme.

CONCLUDING REMARKS

We have selected in a library of randomly mutated ArgRS genes mutations lethal for yeast cell growth. Twenty-eight different mutations that impaired enzyme efficiency were isolated. They essentially map two domains in the protein structure: the active-site domain where the arginine substrate is found, and the presumed anticodon-binding domain located near the carboxy-terminal end. All of them affect the catalytic step of the reaction, possibly by destabilizing the transition state of the activation reaction and in affecting substrates recognition properties. On the other hand this work demonstrates the high efficiency of the approach of random mutagenesis associated to a genetic selection to study the function, structure, and stability of proteins.

MATERIALS AND METHODS

Enzymes, chemicals, and oligonucleotides

T7 DNA polymerase, unlabeled deoxyribonucleotides, and dideoxyribonucleoside triphosphates were obtained from Pharmacia LKB (Uppsala, Sweden). Bacterial alkaline phosphatase came from Stratagene Cloning Systems (La Jolla, California, USA). Restriction enzymes were from New England Biolabs (USA), crude tRNA from yeast was obtained from Boehringer Mannheim (Germany), L-[^{14}C] arginine and [^{35}S]dATPαS from Amersham (UK). 5-fluoroorotic acid was from Toronto Research Chemicals (Canada). Oligonucleotides were synthesized by Genset (France) and NAPS (Germany).

Strains, plasmids, *E. coli*, and yeast strains manipulations

E. coli TB1 (F$^-$ *ara* Δ*(lac-proAB) hsdR* (rk$^-$ mk$^+$) *rpsL*(Str^r) [ϕ80, d*lac* Δ *(lacZ)M15*] was used as a recipient for cloning procedures. The haploid *Saccharomyces cerevisiae* YAMB4 (*a/α ura3-52 lys2-801^{am} trp1-Δ63 his3-Δ200 leu2-Δ1 ade2-Δ450 ade3-Δ1483*) was used for the disruption of the *RRS1* gene and to select the inactive ArgRS. The bacterial and yeast strains were grown and transformed according to standard procedures (Ausubel et al., 1987).

Plasmid pTrc99B was used to express the mutants of yeast ArgRS in *E. coli* (Aman et al., 1988). Plasmid pAL4, used to rescue the ArgRS-disrupted strain, was constructed by cloning the 2.7-kbp DNA fragment encoding *RRS1* into pALR10 (pUN50-*ADE3*); the resulting plasmid was Ura$^+$, Ade3$^+$, RRS1$^+$. pRS314-*RRS1* was constructed by cloning the same

RRS1 DNA fragment into pRS314 (Sikorski & Hieter, 1989); the resulting vector was Trp^+, $RRS1^+$.

In situ disruption of *RRS1*

The 1.15-kbp *Sty*I fragment corresponding to 383 internal amino acids residues from *RRS1* was replaced by the 1.8-kbp *HIS3* fragment in the pRS414-*RRS1* vector. This new plasmid served as the source of the 3.3-kbp DNA fragment with the disrupted *RRS1* gene. The one-step gene disruption procedure (Rothstein, 1983) was used to substitute the Δ*rrs1::HIS3* mutant gene for the wild-type *RRS1* gene in the diploid yeast strain YAMB4. His^+ colonies were selected and induced to form spores on sporulation plates. Then tetrades were dissected with a Singer apparatus and haploids were plated on YPD plates. If *RRS1* codes for an essential gene, each tetrad should have only two viable spores, both being auxotrophic for histidine. Only two viable His^- spores were obtained, showing the essential character of *RRS1*. Then, the diploid colonies showing the segregation 2:2 were transformed by plasmid pAL4 (Ura^+, $Ade3^+$, $RRS1^+$) and again sporulation was induced. Dissection showed four viable haploids per tetrad, a result of the complementation by the plasmidic-encoded ArgRS. We selected the His^+, red and $5\text{-}FOA^S$ haploids and confirmed the integration event by PCR analysis of the genomic DNA. The $Sect^+$ character, resulting from the introduction of another plasmidic *RRS1* gene, was tested after introduction in *trans* of pRS314-*RRS1*. The resulting haploid strain YAMB4-disrupted for *RRS1* was designated YAL4.

Mutagenesis and selection

To generate a library of lethal *RRS1* genes we randomly mutated the pRS314-*RRS1* DNA by hydroxylamine according to Rose & Fink (1987). Then, 50 ng pRS314-*RRS1* were used to transform the YAL4 strain on minimal medium supplemented with adenine (limiting concentration of 2 $\mu g \cdot mL^{-1}$), with histidine (20 $\mu g \cdot mL^{-1}$), with uracil (20 $\mu g \cdot mL^{-1}$), with lysine (20 $\mu g \cdot mL^{-1}$), and with leucine (60 $\mu g \cdot mL^{-1}$). The concentration of 50 ng was determined in order to obtain 300–500 colonies per 88-mm-diameter petri dishes. After 72 h incubation at 30 °C, the Trp^+ red ($Sect^-$) colonies were isolated and screened for 5-FOA resistance. The Trp^+ $5\text{-}FOA^S$ colonies were selected and were expected to contain an inactive ArgRS gene in the pRS314 plasmid.

Overexpression of the different ArgRS

The native ArgRS gene was cloned as a *Nco*I-*Xho*I DNA fragment containing the entire open reading frame plus 400 nt downstream of the TAA codon. The resulting DNA fragment was cloned into the multiple cloning site of pTrc99B (Sissler et al., 1997). The overexpressed ArgRS is a nonfused protein presenting the authentic amino acid sequence. The recipient strain TB1 was grown to $A_{700} = 0.5$ and then induced by adding IPTG to a final concentration of 0.5 mM. After 12 h of induction, the cells were harvested by centrifugation and washed in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). The moderate level of *RRS1* expression was improved by cotransformation with pSBETa, a vector carrying the *E. coli* *argU* gene (Schenk et al., 1995). This gene encodes the rare $tRNA^{Arg4}$, decoding the AGA and AGG arginine codons rarely used in *E. coli*, but used at much higher frequencies in eukaryotic coding sequences (72% of the arginine codons are AGG or AGA codons in *RRS1*). The presence of the "helper" tRNA in the *E. coli* cells increases the level of expression and solubility of *RRS1*.

The mutated *RRS1* alleles were introduced into pTrc99B by replacement of the 1.9-kbp DNA fragment *Eco*RI-*Xho*I of the native *RRS1*. Enzyme extraction and purification to homogeneity was essentially performed according to the protocol established for AspRS (Eriani et al., 1993), except that the ArgRS protein was eluted from the hydroxyapatite column by only 150 mM potassium phosphate buffer, pH 7.5.

Measurement of kinetic parameters

The aminoacylation mixture contained 100 mM HEPES (sodium salt), pH 7.5, 30 mM KCl, 0.1 mg/mL bovine serum albumin, 5 mM glutathione, 10 mM ATP, 50 μM L-[^{14}C] arginine (25–350 Ci/mol), 10 mM $MgCl_2$ and 10 μM of pure $tRNA^{Arg}$ or 200 μM of unfractionated tRNA. The reaction was initiated by the addition of enzyme and conducted at 37 °C. At varying time intervals (usually 1 min), aliquots of 20 μL were spotted onto Whatman 3MM discs that finally were measured for radioactivity. For ATP and $tRNA^{Arg}$ K_m measurements, the substrates' concentrations were varied from 0.25 to 2 K_m values.

For the ATP-PPi exchange reaction, the reaction mixture contained 100 mM HEPES (sodium salt), pH 7.5, 10 mM $MgCl_2$, 2 mM [^{32}P]PPi (1–2 cpm/pmol), 5 μM $tRNA^{Arg2}$ (purified according to B. Delagoutte and J. Cavarelli, in prep.), 2 mM ATP, 15 mM arginine, 10 mM KF. The reaction was started by enzyme addition and after various incubation times at 37 °C, 20 μL aliquots were measured for [^{32}P]ATP formation. For arginine K_m measurements, the arginine concentrations were varied from 0.25 to 2 K_m values.

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